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## Identification of New Inhibitors of Human Low Molecular Weight Protein Tyrosine: Phosphatase Isoform B via Virtual Screening

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Identification of New Inhibitors of Human Low Molecular Weight Protein Tyrosine

Phosphatase Isoform B via Virtual Screening

An Honors Thesis

In Partial Fulfillment

of the Requirements for Distinction

in the Department of Chemistry

By Christopher Seiler

May 1, 2012

The Identification of Novel Inhibitors of Low Molecular Weight Protein Tyrosine  
Phosphatase Isoform B via Virtual Screening  
Christopher Seiler  
Edward McIntee, Henry Jakubowski, Leo Seballos

### Abstract

The human low molecular weight protein tyrosine phosphatase (LMW-PTP) has been identified as a target for inhibition as its overexpression leads to metastatic transformation and invasivity in several human cancers such as breast, colon, bladder, and kidney. Extra precision docking with Glide on LMW-PTP isoform B was used to virtually screen 1356 compounds from the National Cancer Institute (NCI) diversity set II for new potential inhibitors. Twenty-one compounds identified as potential inhibitors by *in silico* screening methods were acquired from NCI. The compounds were kinetically analyzed on LMW-PTP isoform B to validate their selection as potential inhibitors. The best fit inhibition constants for the selected compounds were determined from *in vitro* kinetic experiments. The results of the kinetic assay and inhibition constants are reported and discussed.

### Introduction

Post-translational phosphorylation of proteins is a reversible modification that is critical for control of cellular functions. The phosphorylation of tyrosine residues is a ubiquitous control mechanism for regulation and signaling in eukaryotes. The intricate regulation of these proteins is dynamically controlled by phosphorylation of tyrosine side chains by protein tyrosine kinases (PTKs) and dephosphorylation by protein tyrosine phosphatases (PTPs). The regulated activity of PTKs and PTPs are vital to cell processes such as signaling, growth, migration, and gene transcription.<sup>1</sup>

PTPs can be divided into sub-groups including classical pTyr specific, dual specificity, Cdc25, and low molecular weight phosphatases.<sup>2</sup> These PTPs all share a common active site phosphate binding loop (P-Loop) consisting of CX<sub>5</sub>R(S/T) but differ in otherwise in sequence and specificity. The low molecular weight PTPs have emerged as novel drug targets for their involvement in metabolic and tumor regulation.<sup>1</sup>

The human low molecular weight protein tyrosine phosphatase (LMW-PTP) exists in two active isoforms, A and B, and two catalytically inactive variants, isoform C and SV3 (splicing variant 3). The two active isoenzymes are made by alternate splicing of transcribed RNA causing isoform A to differ in a loop comprised of amino acid residues 40-73 that adjacent to the catalytic site.<sup>1,5</sup> The variation determines specificity for the isoenzymes.<sup>1</sup>

Human low molecular weight protein tyrosine phosphatase (LMW-PTP) is active downstream in the signaling pathways of growth factors and has been linked to cellular transformation.<sup>2-5</sup> LMW-PTP mRNA and protein levels were significantly increased in human breast, colon, bladder, and kidney tumor samples.<sup>6</sup> Overexpression of LMW-PTP in tumor cells is enough to induce neoplasticity in nontransformed cells.<sup>1</sup> Additionally, high levels of LMW-PTP are generally prognostic of a more aggressive cancer.<sup>1,8</sup>

The oncogenic potential of LMW-PTP is closely related to an endogenous substrate, the ephrin type A2 (EphA2) plasma membrane receptor.<sup>1</sup> This EphA2 tyrosine kinase receptor activity is strongly associated within human cancers, contributing to the invasivity of prostate, kidney, and lung cancers. The ligand-independent stimulation of EphA2 increases cellular migration and progression of tumors.<sup>7</sup> The overexpression of EphA2 is associated with increased expression of LMW-PTP which also regulates its activity through dephosphorylation.<sup>1</sup> When associated with EphA2, LMW-PTP is less available for dephosphorylate p190Rho-Gap, which leads to weakened cell-matrix adhesion. This reduced activity on the p190Rho-Gap leads to weakened cell-matrix adhesion.<sup>6</sup> The Rho GTPases are instrumental in the regulation of cytoskeletal reorganization driving translocation.<sup>7</sup>

It has been demonstrated that diverse molecules modulate the activity of LMW-PTP, including flavonoids, quercetin and morin, adenine, and purines.<sup>9-12</sup> Attempts to synthesize inhibitors of LMW-PTP have been impeded by insufficient data about the inhibitor – protein interactions.<sup>13</sup> Although a known inhibitor of LMW-PTP, pyridoxal 5'-phosphate (PLP) displays the tight binding ( $K_{is} = 7.6 \mu\text{M}$ , pH 5) necessary for good inhibition, it is also a necessary cofactor for other enzymes, and thus lacks the specificity required.<sup>14</sup> Two inhibitors for bovine-LMW-PTP (which has 94% sequence homology with the human enzyme), ptp-043 and ptp-194, have similar  $K_{is}$  values ( $11 \pm 2 \mu\text{M}$  and  $9 \pm 3 \mu\text{M}$  respectively).<sup>15</sup> These compounds were discovered through virtual screening of the Chem Div collection (version 2004) via Autodock 3.0.<sup>15</sup> The search for novel inhibitors of LMW-PTP has been aided by virtual screening of compound libraries.<sup>8,15,16</sup>

To increase the speed of discovery, the publicly available National Cancer Institute's (NCI) Diversity Set II was virtually screened *in silico* using Glide.<sup>8</sup> Those compounds which showed the highest *in silico* binding affinity were obtained from the NCI/DTP Open Chemical Repository, and tested for inhibitory activity *in vitro* with LMW-PTP isoform B. The most promising target compounds will be used as scaffolds for optimizing inhibition.

## Results and Discussion

The *in silico* screening of the National Cancer Institute's Diversity Set II led to the selection of 24 compounds for *in vitro* analysis. Given the small amount of sample (5 mg), initial analysis of inhibition was conducted at a single final inhibitor concentration (450  $\mu\text{M}$ ) (single point assay, see supplemental materials) Promising candidates were then tested in either a multiple-point micro or macro assay, depending on the amount of inhibitor available. For solubility reasons, two compounds (639174 and 62840) were tested at a lower single concentration but showed little inhibition in micro assays ( $K_i = 538 \mu\text{M}$  and 3.30 mM, respectively). Nine compounds (107022, 76988, 182400, 156563, 38042, 82560, 77468, 19803, and 7578) showed inhibition constants less than 450  $\mu\text{M}$  at pH 5.5. These were purchased from Sigma-Aldrich and were then tested further. The results of single point test, micro and macro assays are summarized in table 1.

Five of the inhibitors (107022, 76988, 182400, 156563, and 38042) showed strong inhibition, with ( $K_i$  values less than 100  $\mu\text{M}$ .. The structures of these inhibitors are shown in Figure 1. Compounds 107022 ( $K_{is} 10.8 \pm 1.0 \mu\text{M}$ ) and 76988 had and ( $18.8 \pm 2.1 \mu\text{M}$ ) had inhibition constants similar to that of PLP ( $K_{is} = 7.6 \pm 1.8 \mu\text{M}$ ). These compounds interacted with the P-loop through highly oxygenated ring systems

(figures 2 and 3 respectively). Two compounds (156563 and 38042), interacted with the P-loop through an anionic carboxylate (figures 4 and 5). A third compound with the anionic carboxylate is predicted to place the functional group distal to the active site, and direct chlorine atoms toward it instead (figure 6). Two of these (182400 and 38042) gave better fits to the kinetic data as mixed inhibitors. It should be noted that although some of our most effective inhibitors best fit competitive inhibition, others gave best fits to noncompetitive inhibition (182400 and 38042) and uncompetitive inhibition (77468). The structure of compound 77468 docked into the active site is shown in figure 7 for comparison. The pose in figure 7 shows that it is possible for 77468 to bind to the protein while inorganic phosphate is still covalently attached to the active site cysteine and effect inhibition by reducing the rate that phosphate is cleaved by water. Inhibition models other than competitive (as displayed by 182400 and 38042) might arise from different modes of binding of these inhibitors with LMW-PTP.

All of the top five inhibitors have rings which bind in the active site. The top two compounds, 107022 and 76988, are shown to use their highly hydroxylated rings to interact with Arg 18 in the P-loop and a proximal Asn 50. The structure of 156563 shares similarity with NSC 45576 (Figure 1), which was included in Diversity Set I and had an  $IC_{50}$  of 3.9  $\mu$ M at pH 5. These two compounds exhibit similarities in the constraint of the double bonded nitrogen and the oxygenated fused ring system in the active site. A key difference between the two molecules is the type of interaction with the protein. 45576 uses a sulfonate, while 156563 utilizes a carboxylic acid. The structure of 76988 resembles the flavonoids, quercetin and morin, previously reported to activate and inhibit LMW-PTP respectively.

## Conclusion

The *in silico* screening of the NCI Diversity Set II was conducted with Glide for LMW-PTP isoform B. The potential inhibitors from the *in silico* screening were analyzed by *in vitro* kinetic assays. Nine of 24 (37.5%) selected screened inhibitors displayed  $K_i$  less than 450  $\mu$ M, while five (20.8%) had  $K_i$  less than 100  $\mu$ M. The compounds 107022 and 76988 have  $K_i$  values similar to that of PLP, a nonspecific cellular inhibitor and as such might prove to be useful candidates for further investigation. The screening of diversity set II has produced new scaffolds for optimization. Additional testing to gauge the specificity of these new candidates for LMW-PTP would be useful.

Although the screening of Diversity Set II identified inhibitors for LMW-PTP with  $K_i$  less than 100  $\mu$ M, the screen also produced compounds which had  $K_i$  in the millimolar range. The origin of the wide range of results is unclear but indicates that more information about small molecule interactions with LMW-PTP is needed.

## Acknowledgements

The authors would like to acknowledge the support of the National Cancer Institute. We additionally thank the Minnesota Super Computing Institute, the College of St. Benedict and St. John's University for facilities, equipment, and support of the staff.

Table1: Experimentally determined inhibition constants of compounds for isoform B.

Compound (NSC#)	Competitive $K_{is}$ ( $\mu$ M)	Uncompetitive $K_{ii}$ ( $\mu$ M)	Mixed $K_i$ ( $\mu$ M)
107022	$10.8 \pm 1.0$		
76988	$18.8 \pm 2.1$		
182400*			$26.0 \pm 8.0$ $35.0 \pm 6.7$
156563	$33.5 \pm 8.2$		
38042*			$73.2 \pm 29.2$ $108.2 \pm 30.3$
82560	$128.0 \pm 29.9$		
77468**		$121.1 \pm 9.9$	
19803	$125.7 \pm 23.1$		
7578	$161 \pm 19$		
140899	493		
639174	538		
91529	635		
114436	730		
50572	925		
403379	$1.185 \times 10^3$		
81750	$1.563 \times 10^3$		
362639	$1.65 \times 10^3$		
146554	$2.23 \times 10^3$		
58-85-5 <sup>#</sup>	$2.33 \times 10^3 \pm 0.33 \times 10^3$		
134580	$2.78 \times 10^3$		
62840	$3.30 \times 10^3$		
4649-09-6 <sup>#</sup>	$7.19 \times 10^3 \pm 0.66 \times 10^3$		
205842	$17.65 \times 10^3$		
89720	$66.5 \times 10^3 \pm 39.3 \times 10^3$		

\*Best fit noncompetitive inhibition

\*\*Best fit uncompetitive inhibition

<sup>#</sup> CAS number, as no NSC number available

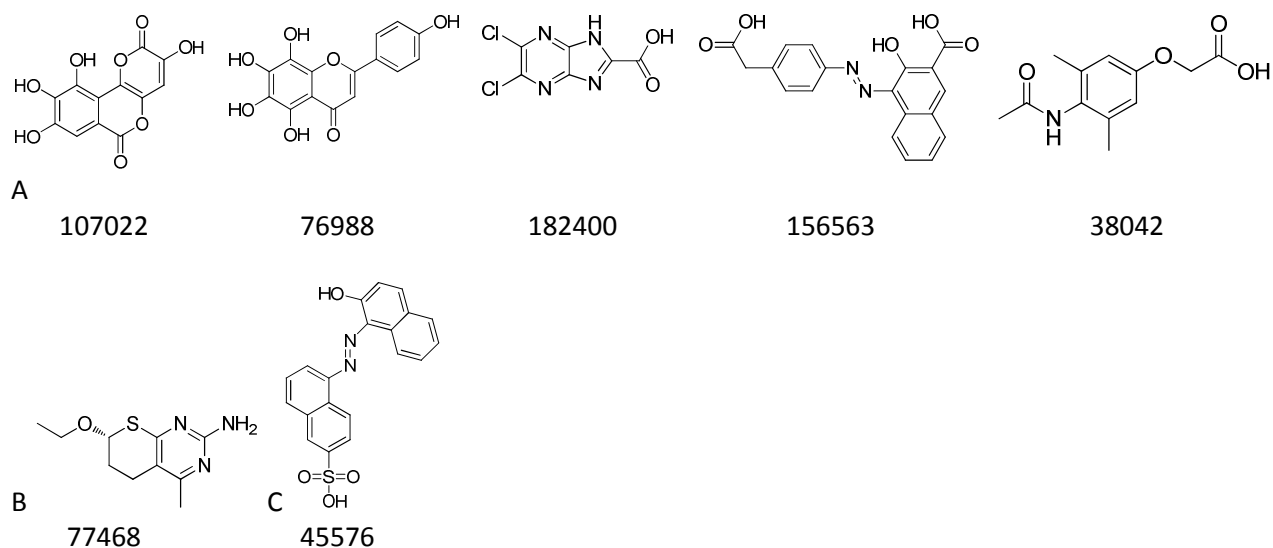


Figure 1. A. Structures of inhibitors having  $K_i$  less than 100  $\mu\text{M}$ . B. Uncompetitive inhibitor 77468. C. Compound 45576, a previously reported inhibitor of LMW-PTP isoform B.

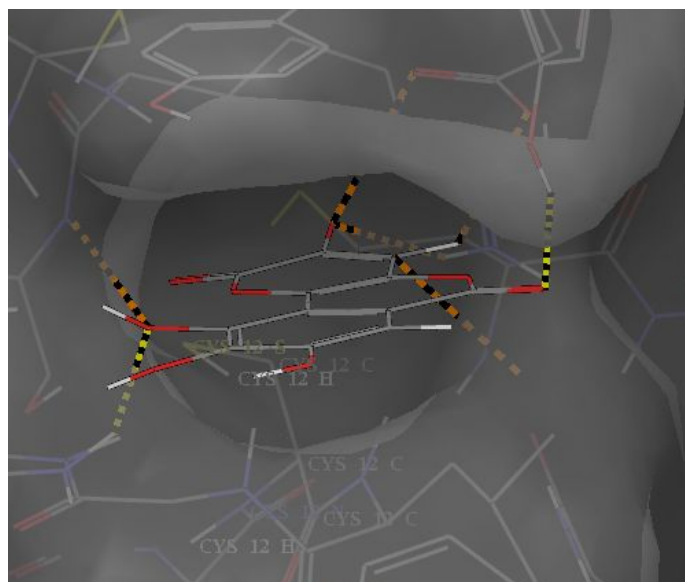


Figure 2. The best fit orientation of 107022 in the active site of 1XWW as predicted by Glide. Also shown are the interactions with active site residues.

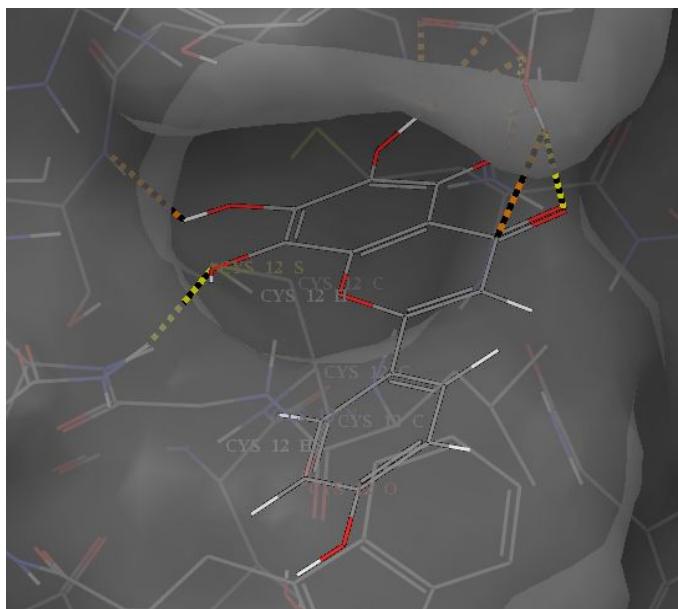


Figure 3. The best fit orientation of 76988 in the active site of 1XWW as predicted by Glide. Also shown are the interactions with active site residues.

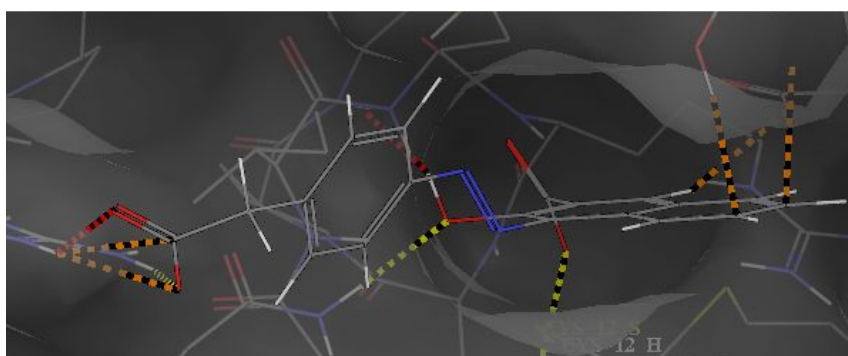


Figure 4. The best fit orientation of 156563 in the active site of 1XWW as predicted by Glide. Also shown are the interactions with active site residues.



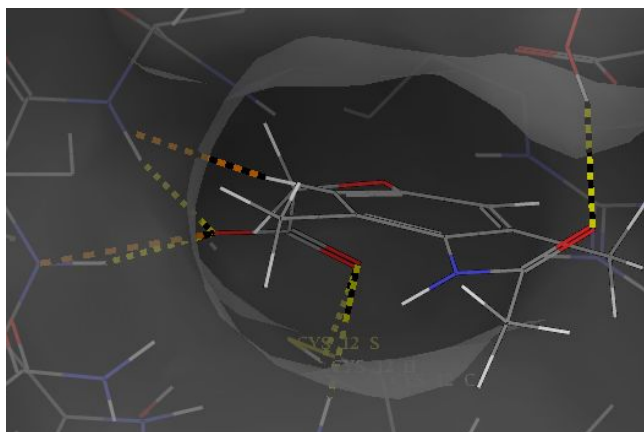


Figure 5. The best fit orientation of 38042 in the active site of 1XWW as predicted by Glide. The best fit was mixed inhibition. Also shown are the interactions with active site residues.

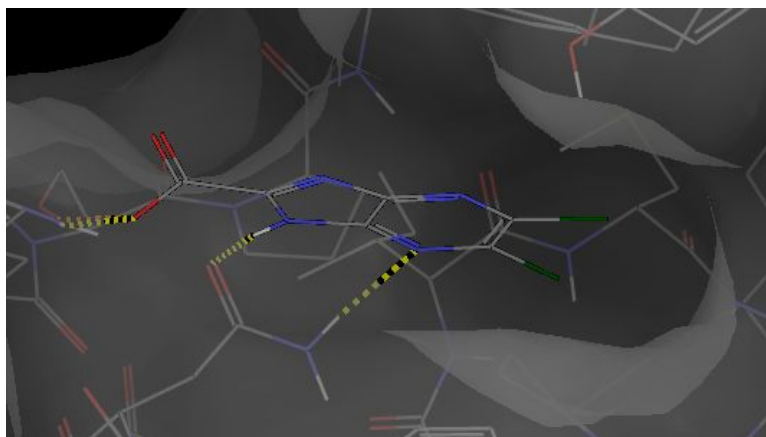


Figure 6. The best fit orientation of 182400 in the active site of 1XWW as predicted by Glide. The best fit was mixed inhibition. Also shown are the interactions with active site residues.

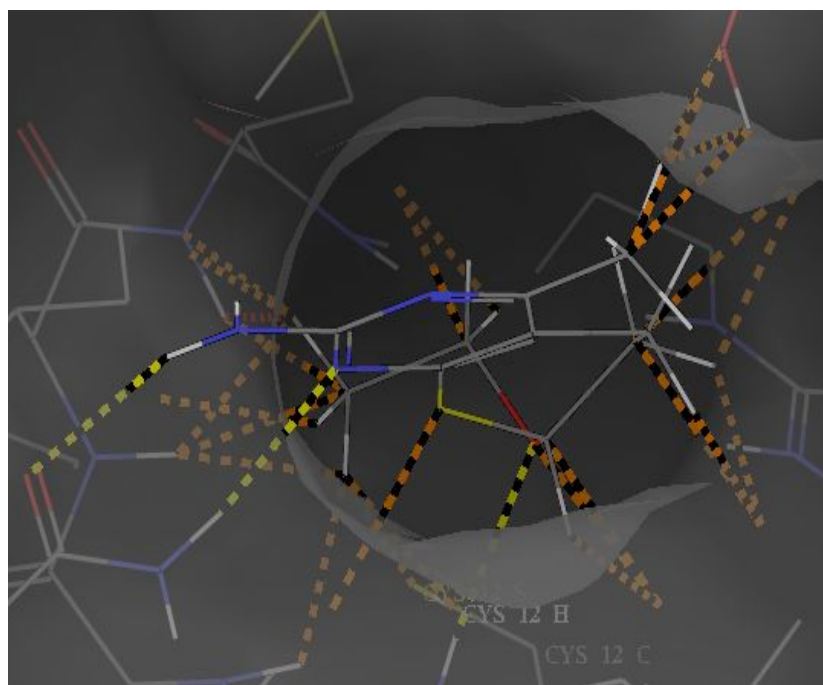


Figure 7. The best fit orientation of 77468 in the active site of 1XWW as predicted by Glide. The best fit was uncompetitive. Also shown are the interactions with active site residues.

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## Supplemental Materials

### Experimental

#### Materials

Compounds with NSC numbers 38042, 205842, 639174, 50572, 114436, 146554, 182400, 140899, 76988, 134580, 81750, 156563, 62840, 82560, 77468, 7578, 91529, 107022, 403379, 19803, 362639 were obtained from the National Cancer Institute Developmental Therapeutics Program. *P*-nitrophenyl phosphate was purchased from Research Organics. Compounds: 58-85-5, 4649-09-6, and 89720, as well as all chemicals for solutions and preparation were purchased from Sigma-Aldrich unless otherwise noted. All absorbance readings were read on a SpectraMax 340PC plate reader.

#### *In silico* screening parameters

The *in silico* screen for potential inhibitors was conducted using the National Cancer Institute's (NCI) Diversity Set II (<http://dtp.nci.nih.gov/>). Using Maestro from Schrödinger, the protein crystal structure (1XWW) was imported from the Protein Data Bank and prepared using the Protein Preparation Wizard. The deletion of waters beyond section is set to 0 and the protein reprocessed. Then the cocrystallized glycerol and sulfate were removed and the pH range was set to  $5.5 \pm 2$ . The protein was refined with exhaustive sampling and optimized. After optimization and deselecting the Hydrogens only option, the protein was minimized. Using Glide, a receptor grid was generated that was centered on the active site cysteine (Cys 12). The ligands were prepared using LigPrep with the force field MMFFs (a pH range of  $5.5 \pm 2$ ), Epik scores, and set to generate at most four per ligand. The docking was done using Glide, with the receptor grid and prepared ligands. Epik state penalties were added, the screen was set to write out at most 10 poses per ligand, and write out residue interaction scores for residues within 20 angstroms. Ligands of interest were chosen based on the most negative docking scores and were then ordered from NCI.

#### Preparation of LMW-PTP isoform B

LMW-PTP isoform B (PDB code: 1XWW) was made in *E. Coli* after transformation of competent cells with a plasmid (provided by David Bernlohr, University of Minnesota) containing human PTP in fusion with glutathione S-transferase under the control of the lac promoter as described.<sup>18</sup> Fusion protein expression was initiated with IPTG. The fusion protein was used for all kinetic assays; stock solution was diluted two-fold in glycerol and stored as a liquid stock at  $-20^{\circ}\text{C}$ .

#### Preparation of Solutions

A 1.0 M sodium acetate/acetic acid pH 5.5 buffer was prepared by dissolving 27.2 g sodium acetate in 200 mL of E-pure  $\text{H}_2\text{O}$ ; 30 mL of 1.0 M acetic acid (5.7 mL glacial acetic acid/100 mL) was added and the pH adjusted accordingly. *P*-nitrophenyl phosphate (pnpp) solution (50.0 mM), was prepared by adding 0.928 g to 50 mL  $\text{H}_2\text{O}$ . This stock solution was diluted to make substock solutions for enzyme assays of 10.0 mM, 5.0 mM, 2.5 mM, 1.25 mM, 0.800 mM, 0.625 mM, 0.500 mM, 0.374 mM, and 0.300 mM. Potassium hydroxide solution, 0.5 M, was prepared using 14.03 g KOH in 500 mL of  $\text{H}_2\text{O}$ . Pyridoxal 5'-phosphate (PLP) solution, 0.263 mM, was prepared by dissolving 6.5 mg in 100 mL  $\text{H}_2\text{O}$  and diluted 1/10 and 1/20 to 26.3  $\mu\text{M}$  and 13.15  $\mu\text{M}$  respectively. PLP and substrate solutions were stored in  $-20^{\circ}\text{C}$

freezer. Inhibitors utilized in the micro assay were dissolved in DMSO to create solutions that had an approximate initial concentration of 18 mM.

#### Enzyme Assay

Enzyme assays used to determine kinetic parameters were conducted by adding specified amounts of a stock 1.0 M sodium acetate/acetic acid buffer solution, H<sub>2</sub>O, and p-nitrophenyl phosphate (pnpp) at final concentrations in the assay mixture of 2.0 mM, 1.0 mM, 0.5 mM, 0.25 mM, 0.16 mM, 0.125 mM, 0.10 mM, 0.075 mM, and 0.06 mM) to microcentrifuge tubes. Enzyme was added at time intervals to initiate the reaction. After fifteen minutes, aliquots were removed to a 96-well plate containing KOH (0.5 M, 200  $\mu$ L) to quench the reaction. A<sub>405</sub> values were corrected for absorbance at each substrate concentration in the absence of enzyme.

The appropriate enzyme dilution was determined in advance by making serial dilutions of the enzyme (glycerol stock stored at -20<sup>0</sup>C) and using the dilution which gave under specified test conditions an A<sub>405</sub> of 0.120 in the plate reader. This dilution, used in all further assays, gave linear changes in concentration of product over the time course chosen for inhibition studies, ensuring that quenched single point absorbance values reflected the initial rates. Initial velocities were also directly proportional to enzyme concentration, indicating the validity of the enzyme assay.

Validation of the enzyme assay was also conducted using PLP as a known inhibitor. PLP in our assays was determined to be a competitive inhibitor with a K<sub>is</sub> of 3.73  $\pm$  0.39  $\mu$ M, in accord with published values. Enzyme assays 2.5% DMSO did not affect the assay results, showing that DMSO used to dissolve the inhibitors had no effect on the enzyme assay. The activity of the glycerol enzyme stock was measured daily as a quality control measure. Activity varied minimally and randomly over a several month period.

#### Scaled Enzyme Assays:

Given the small amount of sample received from NCI, several different sets of assay conditions were used to determine if the samples inhibited the enzyme (single point assay) and then to model the inhibition and determine kinetic parameters (macro and micro assays). These include single point, macro, and mini assays.

For the single point assay (conducted at one substrate concentration), duplicate reactions (V<sub>final</sub> = 100  $\mu$ L) containing inhibitor (18 – 0.36 mM, 2.5  $\mu$ L), H<sub>2</sub>O (62.5  $\mu$ L), acetic acid/acetate buffer solution (1.0 M, 5  $\mu$ L), and pnpp (1.25 mM, 20  $\mu$ L) were performed. At 30 second intervals, enzyme (10  $\mu$ L) diluted in 1.0 M acetic acid/acetate buffer solution was added and allowed to react for 15 minutes. The reaction was quenched by adding KOH (0.5 M, 150  $\mu$ L) to the reaction mixture and 240  $\mu$ L of the quenched reaction mixtures were added to a 96-well plate for analysis.

The macro assay was used for compounds purchased from Sigma. The reactions mixtures (V<sub>final</sub> = 500  $\mu$ L) contained acetic acid/acetate buffer solution (1.0 M, 50  $\mu$ L), H<sub>2</sub>O (330  $\mu$ L), varying concentrations of pnpp (100  $\mu$ L) in the absence of inhibitor (330  $\mu$ L water) and presence of two different fixed concentrations of inhibitor (0.5 mM to 10.0 mM, 330  $\mu$ L) whose concentrations were determined by the

single point assay. Measurements in the presence and absence of inhibitors were done in the same assay. At 30 second intervals, enzyme (20  $\mu$ l) was added to the reaction mixture which was allowed to react for 15 minutes. The reaction was quenched by removing 100  $\mu$ L of the reaction mixture to a 96-well plate containing 150  $\mu$ L of 0.5 M KOH.

The micro assay was used for compounds received from NCI after the single point test. The reaction mixture ( $V_{\text{final}} = 100 \mu\text{L}$ ) contained acetic acid/acetate buffer (1.0 M, 5  $\mu$ L),  $\text{H}_2\text{O}$  (62.5  $\mu$ L), varying concentrations of pnpp, and inhibitor (18.0 to 0.36mM, 2.5 $\mu$ L). Inhibition analysis was conducted simultaneously with two different concentrations of inhibitor based on calculations from the single point test. Measurements in the presence and absence of inhibitors were done in the same assay. At 30 second intervals, enzyme (10  $\mu$ l) was added to the reaction mixture which was allowed to react for 15 minutes. The reaction was quenched by removing 100  $\mu$ L of the reaction mixture to a 96-well plate containing 150  $\mu$ L of 0.5 M KOH.

#### Analysis using Visual Enzymics©

The inhibition constants ( $K_{ix}$ ) were calculated using Visual Enzymics through the IgorPro interface using the one substrate one inhibitor section of the program. In the data page, the concentration (mM) of the substrates and inhibitor in the reaction mixture were entered along with the velocity of the reaction. The standard deviation and mask were set to 1.  $A_{405}$  values were converted to molarity units using an extinction coefficient and path length for the plate. The data was fit to the equations for competitive, uncompetitive, and mixed inhibition models.